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FLASH CONTROL AND FEMALE DIALOG REPERTORY IN THE FIREFLY *PHOTINUS GREENI*

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Abstract

In the *Photinus greeni* courtship dialog the male emits flashes in pairs (S1S2) about 1250 ms apart at 25°, the pairs recurring every 5 to 7 s. The female answers with one flash, usually about 750 ms after the male's S2 signal (S2R response). S2R latency includes 400+ ms of central nervous delay. Using paired signals of electric light, female responsivity to stimulation at different frequencies was established and excitatory state modified so that S1Rs (responses to the first flash of signal pairs), and spontaneous flashing, became more frequent. S1Rs have a longer latency than S2Rs.

Flash timing was examined for presumed neural noise, statistical and individual variation, persistence, response cycling, hyperexcitation, fatigue, and habituation. A model central neural flash-control mechanism, based on an excitability transient rising from a resting level to a flash-triggering level, distinguishes S1R from S2R and accounts for much behavioral timing.

The female clearly has an *input*-timing element, used normally for identifying the male's signal pair. Since females sometimes emit pairs of spontaneous flashes at about the same average interflash interval as the male's, it is suggested that her timer may, under stress, assume the *output*-timing role normal in the male.

Introduction

Because of the unique time-coded flashing in the sex-recognition dialogs of many lampyrid fireflies and the ease with which their signals can be simulated, several courtship protocols have been studied intensively. Field investigations have revealed species-specificity and great diversity in coding, while signal simulation has made it possible to quantify visual, timing, and response parameters and relate them to rhythm generation and other processes in the firefly central nervous system.

In *Photinus greeni* (Lloyd, 1969) the flying male's periodic advertising luminescence is a pair of flashes, or "phrase" (Lloyd, 1966), rather than a single flash. The stationary female times her flash from the second signal (S2) of properly timed flash pairs (S1S2). The dialog is a particularly attractive study system because both intra- and inter-phrase timing can be varied experimentally.

Buck and Buck (1972) reported that at 27°C the flashes in the male's phrase had a duration of 100 ms and were about 1300 ms apart. Phrases were repeated about every 5 s while the male was flying and, less regularly, after he had landed and was walking toward the female. The female answered with a 200 ms flash after the male's second flash. The average response latency to simulated male phrases was about 850 ms. Like the females of many other *Photinus* fireflies, the *P. greeni* female does not require successive male signals to be presented rhythmically, tolerates much variation

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Abbreviations: S1R, response to first flash of stimulus pair; S2R, response to second flash of stimulus pair; S1S2, interval between flashes of stimulus pair; SP, spontaneous flash.

in signal duration and intensity, and twists her abdomen when flashing, aiming her light toward the male.

Using manually controlled simulations of the male's stimulus phrase (S1S2), Buck and Buck (1972) showed that the acceptable intra-phrase interval could differ substantially from female to female and that the range was wider than that actually used by males in the field. They noted an occasional response by the female to the first flash of the male's phrase pair (S1R) but regarded such irregularities as rare. They also concluded that the female, after being stimulated by S1, is refractory to further photic input for about a second.

In several firefly species the delay between light reception by the eye and the occurrence of the response flash much exceeds the delay between direct electrical stimulation of eye or brain and the resulting flash (Case and Buck, 1963; Magni, 1967; Hanson *et al.* 1971; Buck *et al.*, 1981B). Only a few milliseconds could reasonably be allocated to visual processes (Case, 1984). The interval between arrival of the visual message in the brain and the departure of the motor neural message from the brain to the (abdominal) light organ thus constitutes a specific central nervous delay, which is evidently by-passed by electrical stimulation. In the *P. greeni* female the electrical latency is 250–300 ms (Case and Buck, in prep.) so at least 400 ms of the photic latency (range 650–950 ms) must be central delay.

It is very helpful, in experimenting with photic response, that (1) the nearly hemispherical eye is very sensitive to light normal to any part of the corneal surface, (2) both sexes are extremely tolerant of differences in signal flash intensity (as expected from the fact that they court over a distance range of several meters; Buck and Buck, 1972: Case and Buck, 1973), and (3) firefly dialog exchanges tend to be all-or-none: the individual either responds fully to the signal or does nothing. In the *P. greeni* female the range of acceptable photic stimulus intensities is about 10^4 (Case and Buck, in prep.). Unrestrained specimens can thus be used in behavioral work with good assurance that all signals of moderate intensity will be seen except those from directly behind.

In the present laboratory investigation we recorded females' dialogs with artificial signals of controlled intensity, duration, number, and timing and analyzed response latency and sequence in relation to phrase repetition frequency and pattern. We also studied spontaneous flashing and the usually infrequent and seemingly anomalous response to the first flash of the stimulus pair (S1R). A main objective was to explore timed elements in flash control and photic dialog in intact, unrestricted females as indications of neural circuitry involved.

The species-specificity of firefly dialog led historically to the impression that each photic code is rigid and invariant. *Photinus macdermotti* shares with *P. greeni* a code involving paired male flash signals and a singly flashed female reply. In this species, differences between signal timing by answered and unanswered males and other variable behaviors have been reported (Lloyd, 1969, 1981, 1984; Carlson *et al.*, 1976, 1977). Such observations appear to widen the potentialities of firefly communication systems. Hence, in the present analysis of flash timing in *P. greeni* we paid particular attention to the lability of female photic behavior and to behaviors reported to exist in *P. macdermotti.*

Materials and Methods

Females of *Photinus greeni* were collected between 8:30 and 11:00 p.m. EDT at several sites in Woods Hole, Massachusetts, during June, July, and August of six summers, mostly 1972–1974. Animals were located on perches in vegetation by their

replies to paired flashlight signals. Specimens were stored in dim room light in 35 × 65 mm plastic vials with white snapcaps, humidified with paper toweling dampened with dilute sucrose solution, and used for up to 10 days. Prior to experimentation each female was dark-adapted for at least 15 minutes and tested repetitively with paired signals until response was stabilized. Experiments were run between 6:00 a.m. and 11:00 p.m.

Usually four females were tested simultaneously in a light-tight box 1 m long in which a female was stationed in each of four 7 × 10 × 15 cm compartments at one end, shielded from the other females by light baffles (Fig. 1). Controlled flashes from one or from two Sylvania 911 glow modulator lamps were conducted singly or consecutively by light guides to a diffusing surface at the other end of the box so as to illuminate each compartment equally. The response flashes of each female were detected separately via an RCA 1P21 photomultiplier viewing her chamber from above. Each female was placed in a chamber still in her residence vial, which was inverted so that the white cap would reflect her flashes to the photomultiplier. Durations and times of presentation of glow lamp flashes were controlled by a pair of Grass S44 stimulators.

Temperature in one vial and in the main chamber was monitored via separate thermistors. Temperatures during experimentation varied between 21° and 24°C at the start of a day's work and commonly rose about 1° by the end. In another study we found temperature coefficients of several elements of the female's photic response to be slightly above 2 (Case and Buck, in prep.). Ambient temperatures thus should be considered when measurements from different days are compared or pooled. However, most of our data are from single days (maximum 1° change) and often from

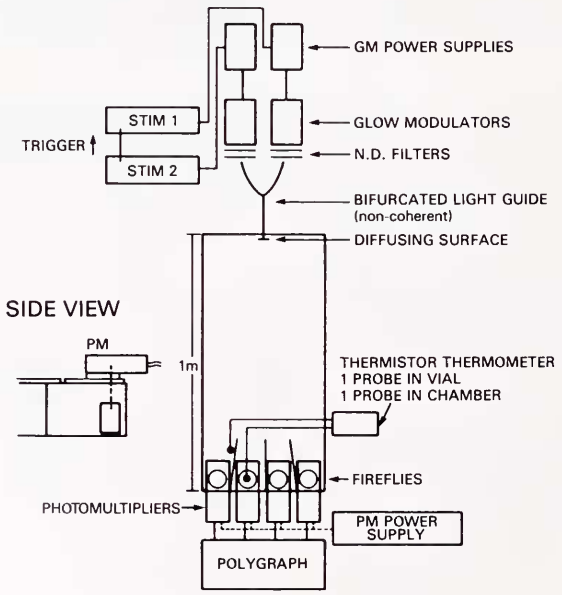

FIGURE 1. Apparatus for inducing and recording photic response. Flashes of glow modulator tube light, with duration, intensity and sequence controlled by physiological stimulators, were delivered to a diffusing surface viewed by 4 in-vial female fireflies, each in a separate chamber, at the other end of the box. Each firefly's light was detected from above by a separate photomultiplier photometer. Output from photometers was recorded on multichannel chart paper.

females in the same run. Also, as shown below, differences between response latencies of different females tested together, or even between means for the same individual at different times in the same experiment were sometimes highly significantly different. We have not normalized the data because we feel it would give a deceptive appearance of uniformity and obscure what we consider an important feature of firefly communication, the variability in timing.

Stimulus artifacts and responses of females were recorded on separate channels of a Grass Model 7C polygraph. Latencies were read to the nearest 5 to 100 ms, as appropriate to chart speeds from 100 mm to 5 mm/s. Timing was from rise point to rise point.

We did not measure flash intensities because of possible variations connected with abdominal aiming (Introduction) and because we often chose recording levels in which the detector was saturated at flash peak in order to get more accurate latencies.

Statistical variation is indicated as mean ± standard deviation (σ), not standard error, and by V, the coefficient of variation.

Results

1. Warm-up behavior

In broadcasting simulated male phrases while collecting females we found that specimens in the field not uncommonly responded to some S1 signals, or even to both S1 and S2 successively. Typically also, females that had been in room light and were then subjected to darkness and exposed to rhythmic S1S2 pairs usually did not respond during the first few minutes and then began an irregular mixture of S1Rs and S2Rs, often interspersed with flashes that occurred 2 s or more after signals. Since data presented below (results, Section 7) showed that exogenously stimulated photic responses occurred only between 600 and 1800 ms post-stimulus, flashes with longer latencies were classified as spontaneous (SP, Fig. 2).

Before females responded regularly they usually required several minutes of stimulation at 20 to 30 s intervals. In 43 randomly chosen runs begun between 7:00 a.m. and 10:00 p.m., the stimulation time necessary to reach sustained S2R responsivity ranged from 5 to 16 minutes ($M = 9.9 \pm 2.9$). In specimens that had been in the laboratory for a day or so there were no marked differences between morning and afternoon experiments. However, even passage of time and repeated presentation of proper signals were not enough to insure response if the female was not receptive. It was noticed repeatedly during video photography (Case and Buck, in prep.) that a freely walking female ignored dialog signals until she had mounted a suitable perch, come to a complete halt, extended her head from beneath the pronotum, and spread her antennae.

2. Stable S2R response

Response-ready females stimulated once every 15 s or less frequently tended to give uniform S2Rs to each consecutive phrase throughout long series, whereas those stimulated every 10 s, or more frequently, often developed response irregularities.

In one typical test series with 4 females exposed simultaneously to one pair of stimulus flashes every 30 to 45 s, two animals missed only a single response each in 156 consecutive cycles and the other two only 19 and 36 (33 at the start), respectively. Flash timing was highly regular, latency V values commonly being as low as 1.5 for 10 consecutive cycles. Each individual appeared to maintain flash intensity within narrow ranges (Fig. 3). Control observations in very dim light showed that responding

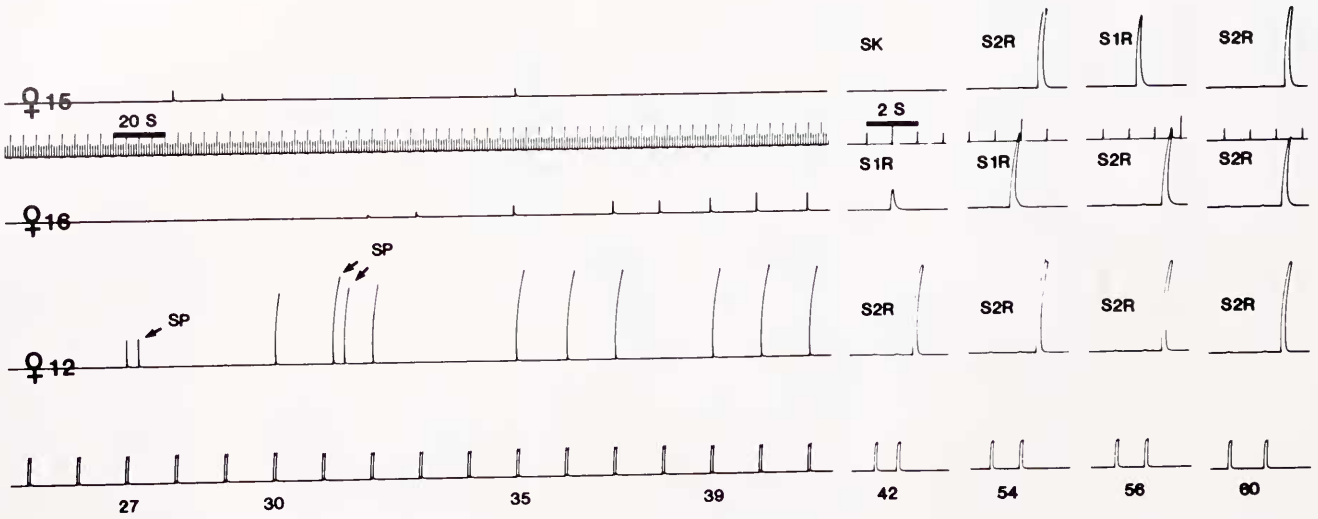

FIGURE 2. Typical flashing during warm-up. Three females were exposed to one phrase pair of 100 ms signals every 18 s (bottom channel). Female 12 (next to bottom channel) responded (S2R) first at cycle 27, 8+ minutes after setup, then to phrases 30, 32 and 35-7, with some interspersed spontaneous flashing (SP) also. At phrase 39, Female 12 began regular 1:1 response. Females 15 and 16 were slower in getting started. After phrase 41, chart speed was increased 10-fold to illustrate skipped responses (SK) and S1Rs still occurring in the Female 15 and 16 traces. All three animals were responding regularly by phrase 60.

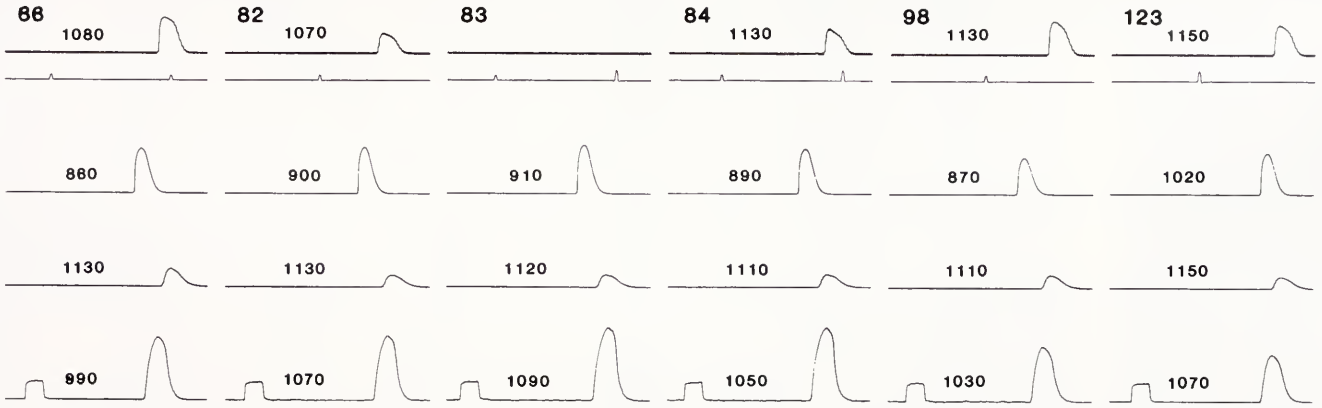

FIGURE 3. Responses of 4 females stimulated once every 30 to 45 s. Six cycles out of several hundreds are shown, with the S2 signal superimposed on the record of the bottom channel female. Response latencies in milliseconds are given to left of flash traces. Note individual constancy (horizontal sequences) and differences (vertical comparisons). Flash skipping was absolute (top female, episode 83). Paired 150 ms signals, 1500 ms apart; 21°; time marks (2d channel) 1 s.

captive females usually did not shift body position for long periods, so it is believed that the apparent uniformity in flash intensity was valid.

Two other points of interest during slow driving were (1) that flash-skipping, if it occurred, was sudden and absolute, with intensity and latency of the first post-skip flash close to those of the flash just preceding the skip (Fig. 3, cycles 82–84, top channel; *cf.* also Figs. 5 and 6), and (2) there were marked and consistent inter-individual differences in latency. In the two most responsive females of the Figure 3 run the mean latencies (S2R) for 100 responses (21°) were 886 ± 27 ms and 1130 ± 38 ms, with Vs of 3, and highly significantly different from each other.

3. Timing latitude in dialog

Figure 4 shows the mean percentages of S2R responses for 13 females presented with 2900 S1S2 stimulus phrases ranging in duration from 550 to 1850 ms. Each phrase was presented two to four times at intervals of 10 to 15 s, followed by another group of phrases of different duration, and so on, some of the females being run again on a different day (total responses 1600). The heavy line shows that intra-phrase durations between 900 and 1400 ms evoked responses more than 50% of the time on the average. The horizontal lines show the ranges of essentially 100% response in the 11 runs evoking more than 70 responses each, with the dashed extensions indicating the shortest and longest phrases to which the particular individual ever responded. As with females stimulated at long intervals (Fig. 3), whenever an animal failed to respond, whether in the high response range or where response was rare, failure was not preceded by dimmer flashes but was sudden and complete as if the flash-triggering process were all-or-none.

Females exposed simultaneously to the same stimulus regimen often exhibited a remarkable degree of individuality. For example, in Table I, which lists the mean response latencies of 3 females during several 12-cycle sequences in a 200-cycle run,

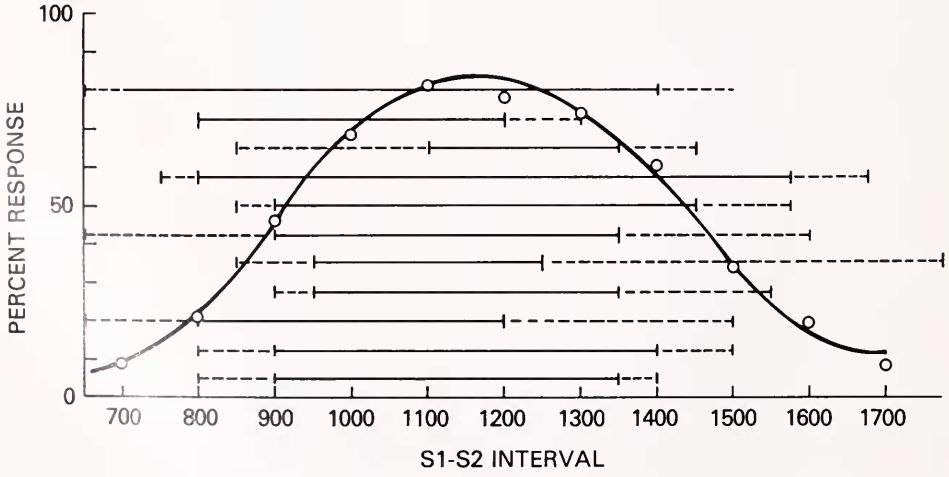

FIGURE 4. S2R percentage in relation to stimulus phrase duration (S1S2). Phrase interflashes between 900 and 1400 ms elicited 50% or higher responses per test (heavy curve). Horizontal lines are the nearly 100% response ranges for 11 runs with more than 70 responses each. Dashed extensions indicate exceptional long and short phrases answered. Thirteen females, 25 runs, 2900 phrases, 1600 responses, stimulus durations 100 ms, 23–25°, not normalized.

TABLE I

Mean response latencies of three females during a run of 200 successive S1S2 stimulus pairs of glow-modulator lamp flashes (25°)

Sequence	Cycles	Phrase presentation order	Female 4	Female 11	Female 14	t (signif.)
1	7–20	970, 1020, 1120, 1220, 1340	751 ± 46	—[1]	663 ± 30	4/14[2]
2	34–45	970, 1150, 1210, 1350	716 ± 71	629 ± 70	641 ± 36	4/11, 14
3	64–77	890, 950, 1040, 1140, 1220	751 ± 56	663 ± 31	628 ± 25	4/11, 14; 11/14
4	117–127	960, 1060, 1140	756 ± 35	645 ± 23	635 ± 37	4/11
5	128–139	1140	735 ± 33	665 ± 18	620 ± 8	4/11; 11/14
6	162–174	850	—[3]	752 ± 54	656 ± 18	11/14
7	185–201[4]	1140	766 ± 55	691 ± 56	653 ± 33	4/11, 14; 11/14
Means[5]			746 49	674 42	642 27	
t				6/2–7[6]	1/3,5	

[1] Female 11 did not begin responding until after the 20th phrase presentation.

[2] "4/14" means that the mean response latency of female 4 differed significantly from that of female 14 by t test at the 5% level or lower.

[3] Female 4 failed to respond to phrases of 850 ms duration. All three females failed to respond to phrases of 750 ms.

[4] 10 ms pulse in this series instead of usual 100 ms.

[5] Periods and standard deviations averaged independently.

[6] "6/2–7" means that the latency in sequence 6 of female 11 differed significantly from runs 2, 3, 4, 5, and 7 of that female.

each individual's mean differed significantly from those of one or both of the other females in nearly every possible pairing (horizontal comparisons). In the within-female comparisons (columns) there was less variation. Both between-female and within-female comparisons seemed independent of whether S1S2 phrase duration was changed frequently (lines 1–4) or held constant (lines 5–7), and of whether the comparison was made early or late in the run. Variability *per se* also differed markedly among females, individual V values ranging from 1.3 (Female 14, sequence 5) to 10 or 11 (Females 4 and 11, sequence 2), with an overall mean of about 5.

No correlation was found between response latency and stimulus phrase duration. Table II gives latencies to all phrases shorter than 980 ms and longer than 1340 ms

TABLE II

Mean response latencies in relation to S1S2 stimulus phrase duration

		Phrases <980			Phrases >1340	
Run	Female	n	M & σ	t	n	M & σ
1	4	34	768 ± 63	0	11	767 ± 90
2	11	44	685 ± 60	1.85	12	644 ± 89
3	14	59	682 ± 50	1.38	9	706 ± 32
4	14	27	726 ± 76	1.83	12	683 ± 47
5	18	12	774 ± 57	2.95	12	702 ± 62
6	9	34	730 ± 50	0	30	724 ± 56
Totals/Means		210	728		86	704

300 responses selected from a total of about 1000 tests on 5 females.
Runs 1–3 from one day's experiment, 4 and 5 from another and 6 from a third.

for 5 females with similar response ranges. There was no significant difference between short and long in 5 of the 6 comparisons, though there were considerable differences between Female 14's runs on different days and large differences between different females in the same run (No. 4 *versus* Nos. 11 and 14 in the first day's run).

4. Frequency effects during rhythmic stimulation

Females stimulated every 10 s or oftener not only responded less consistently than when phrase repetition period was longer (Section 2) but did not respond *consecutively* to stimulus pairs presented more frequently than about once every 4 s. Figure 5A–C shows a continuous record of S2Rs emitted by two females exposed to, first, 6 signal pairs (5 cycles) at 9 s intervals (5A: 3 responses by Female 1, 5 by Female 2); next, 16 pairs at one per 4.5 s (5B: 5 responses by No. 1, 11 by No. 2); and, finally, 12 pairs at one per 3.7 s (5C: 3 responses by No. 1, 6 by No. 2). In 5C, note that Female 2 responded regularly to *every other* pair.

When phrase presentation frequency was increased still further, so that stimulus flashes succeeded each other in an even rhythm (5D), it was, of course, no longer possible to associate responses with particular stimulus flash pairs. Assuming that the standard paired signal format was nevertheless still operating, the responses of Figure 5D were as if every third flash was not seen and flashes 2, 5, 8, etc., functioned as S2s in evoking responses. Runs of up to 12 consecutive "every third" responses were recorded. In all these, flash succession was still limited to a minimum of about 4 s and the interval between last effective S2 and next effective S1 was never shorter than 2.5 s. Also, response flash intensity usually decreased progressively as if the system was being pushed too hard and refractoriness was building up. Thus, in longer series, in which response typically failed at irregular intervals for several cycles at a time, the first flashes after resumption were considerably brighter (Fig. 5D) as if some depressing influence had dissipated. One of the Figure 5D females responded *repetitively* only when driven at intervals of 1125 ms or longer (38 runs) but gave an *initial* S2R (only) in each of 4 runs in a 1070 ms rhythm and of 3 runs at 1000 ms.

In addition to the usually regular S2R responses to rhythmic signal series, there were rare instances of response after the first signal of a series (5E), after the third (5F), after both second and third (5G), and of doubled flashes with the two elements only 300 ms apart (5H).

At rhythmic photic signal presentations faster than one per second, females never responded. One period tested, 580 ms, was interesting because alternate flashes in that rhythm were within the acceptable phrase range ($2 \times 580 = 1160$ ms). The response failure thus indicated that a flash intercalated between S1 and a properly timed S2 was inhibitory (*cf.*, Buck and Buck, 1972).

5. Fluctuating excitability during prolonged driving

Persistent repetitive driving sometimes evoked sporadic and much increased frequencies of the three ordinarily rare events seen in warmup behavior (Section 1): flash skipping, spontaneous flashing, and S1Rs. Flashing series rich in these sporadics sometimes alternated with intervals of very regular response. Figure 6A–F illustrates response heterogeneity during a short portion of a 140 minute run of rhythmic driving. The subject female emitted S1Rs (A), then a few conventional S2Rs (B, cycles 7–10), then primarily S1Rs again (C, D, cycles 20–38), then flashed spontaneously (E), then gave more S1Rs (F) and so on. It might be questioned whether even the rhythmic flashing was actually responsive, but the in-series latencies were clearly non-random (*e.g.*, M $= 1200 \pm 82$ ms in D, cycles 30–36).

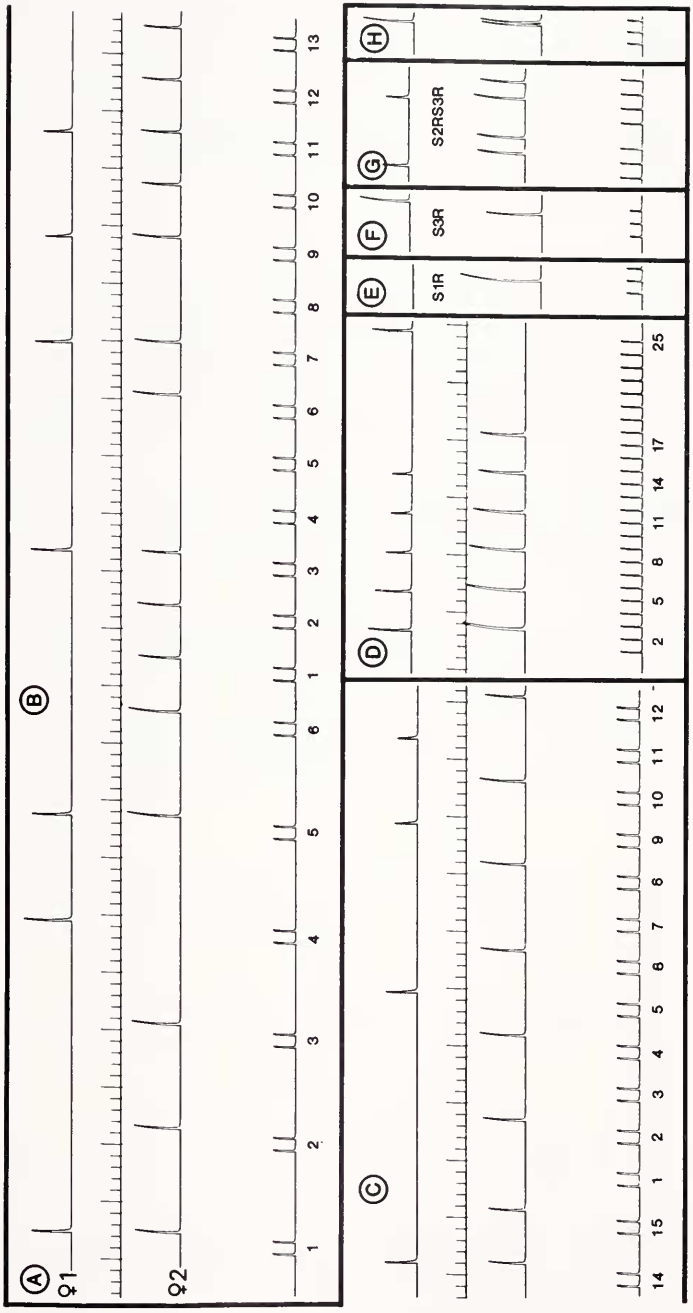

FIGURE 5. Responses of two females to rhythmic photic driving. Runs A through C continuous. A, 5 cycles at one phrase per 9 s; B, 15 cycles at one phrase per 4.5 s; C, 11 cycles at one phrase per 3.7 s. One second time comb (2d channel) applies to all figures. Except for 4 skips, Female 2 (3d channel) responded 1:1 at the 9 s and 4.5 s rhythms. At 1/3.7 s (C) she responded regularly to every other stimulus pair. Female 1 (top channel) responded only about half as often. D, responses to 1200 ms train were given after each third signal, with progressively decreasing intensity. All responses in A–D were S2Rs. Very rarely, a response occurred after S1 (E), after S3 (F), after each of two consecutive signals (G) or as a double flash (H). Different females in each series of D–H and different from those in A–C.

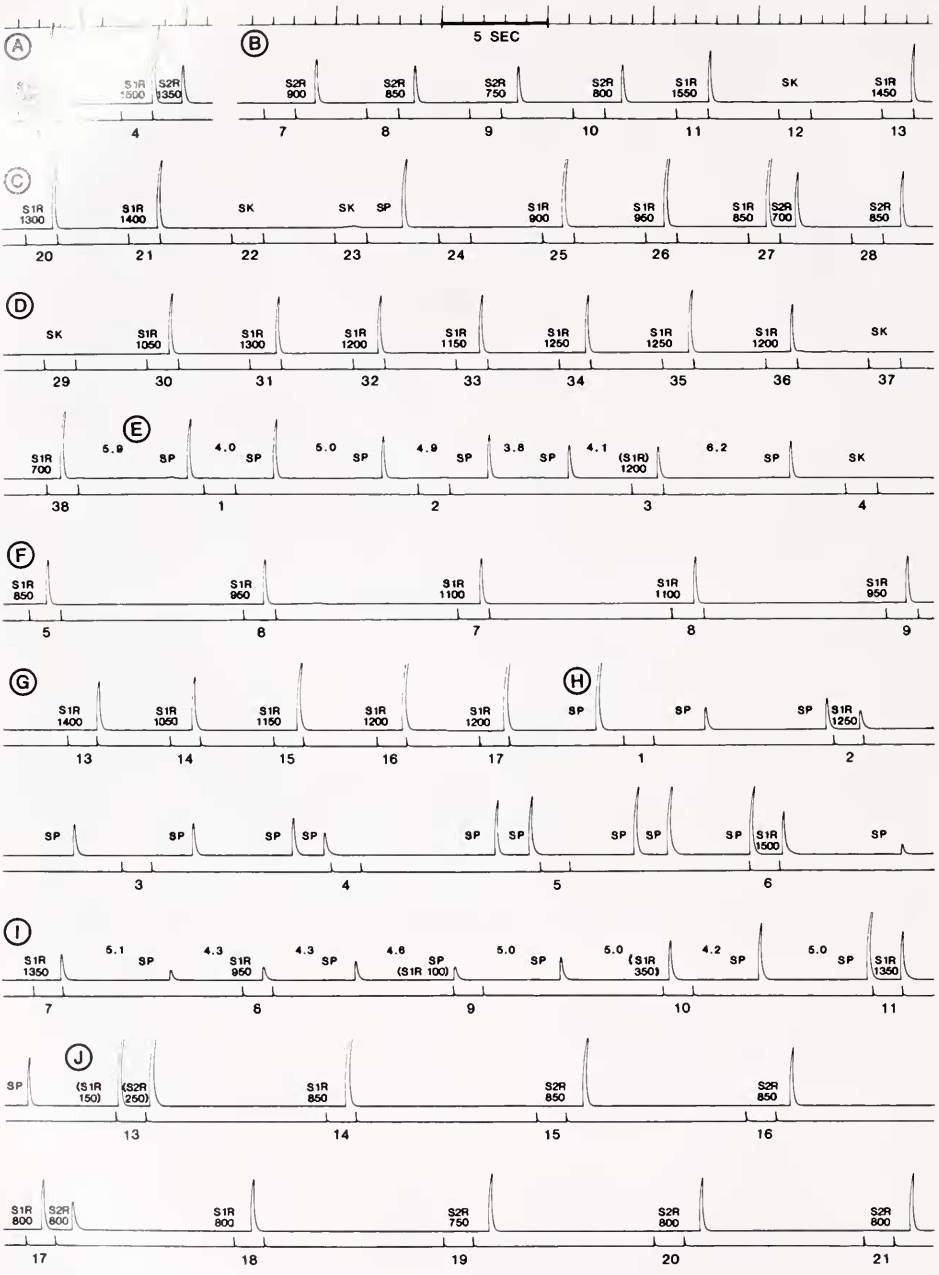

FIGURE 6. Fluctuating response during rhythmic photic driving. A–D, three sequences during 38 cycles of driving at one 1500 ms phrase each 5 s, showing alternating runs of S1Rs (phrases 3, 4, 11–27, 30–38), S2Rs (phrases 7–10, 27–28), skipped responses (phrases 12, 22, 37, etc.) and spontaneous flashes (SP). E–F, 9 cycles of driving at 1/10 s, during which there was first spontaneous flashing (1–4) then regular S1Rs (5–9). Except for indicated omissions, record A–F is continuous. G–J, continuous driving sequence from different part of record of same female, showing S1Rs at one phrase per 5 s (G), followed by single and paired spontaneous flashes (H) and a mixture of S1Rs and S2Rs during stimulation at 10 s intervals (J). Durations of some spontaneous interflashes given in seconds with decimals. Female 62; 22°; signal duration 2 ms. Latency values in parentheses interpreted as spurious or (E3) spontaneous.

We occasionally changed the driving frequency from one phrase per 5 s (A–D) to one per 10 s (E–F) but, as shown by a second sample series from the same run (G–J), there was no correlation between stimulation frequency and type of response. Not only did type of response not necessarily change with the signal, but the female showed herself able to maintain either regular 1:1 S1Rs or S2Rs at either 5 s or 10 s rhythms (*e.g.*, B, cycles 7–10 *vs.* J, cycles 17–21; D, cycles 30–36 *vs.* F, cycles 5–9). We attribute the fluctuating responses to the persistent stimulation. Insight into these behaviors, and implications for flash control mechanisms, were derived from further consideration of the relations between spontaneous flashing and S1Rs (Section 6–8, below, and Discussion).

6. *The S1R enigma*

S1R flashes were generally indistinguishable in intensity and kinetics from S2R flashes of the same animal, and the respective latencies were sometimes in the same range. A low-light video study, to be reported elsewhere, showed that captive females giving an S1R sometimes executed the lantern-aiming movement that almost invariably accompanies the normal S2R. Like spontaneous flashes, S1Rs were usually infrequent in comparison with S2Rs. In more than 9500 box tests during one season there were only 64 S1Rs and 30 instances in which females flashed after both S1 and S2 (S1RS2R) whereas there were several thousand S2Rs; and of 47 females tested only 23 gave any S1Rs. Such evidence suggests that an S1R is due to a central nervous timing perturbation that causes an S1 to be treated as an S2 (see further).

Though most females did not produce enough S1Rs for quantitative comparison with S2Rs of the same individual, a few much-stimulated specimens responded relatively often to the first flash of signal pairs. For example, female 62, featured in Figure 6, gave many S1Rs during the 140 minutes' exposure to 916 phrases (1932 stimulus flashes). Figure 7 shows the frequency distributions of the intervals between each signal and the first following flash. *Taken at face value* there were about 350 S1Rs, with latencies concentrated between 700 and 1800 ms (Fig. 7A), and 250 S2Rs distributed

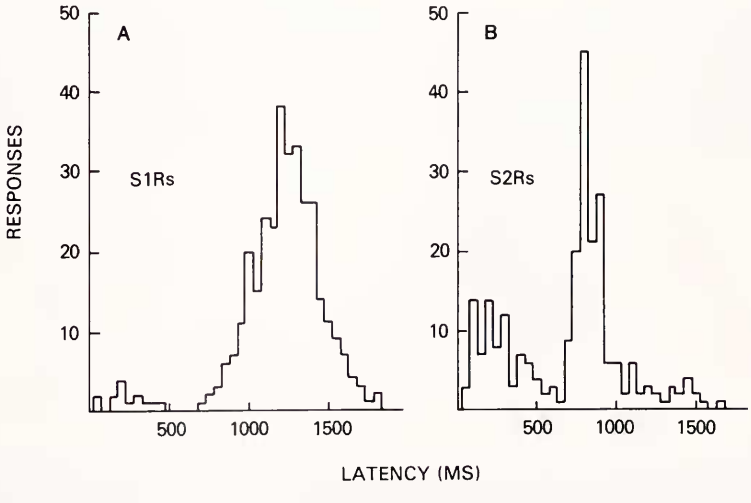

FIGURE 7. Frequency distributions of *prima facie* S1R (A) and S2R (B) latencies of female 62 during 916 cycles of continuous rhythmic driving.

between two populations, one peaking between 700 and 1000 ms, the other concentrated mainly below 300 ms (7B). The overall record included several quite uniform runs of 10 or more consecutive S1Rs in the 1100 to 1200 ms latency range and a like number of runs of 10 or more consecutive S2Rs in the 700 to 850 ms range, so there seems little doubt that there do exist valid photic responses to both S1 and S2. It also seems safe to conclude from Figure 7 that any flash that follows a signal by more than 2000 ms is not a response but a spontaneous emission.

7. Further S1R/S2R interrelations. Implication of spontaneity.

In many experiments, under a variety of conditions, only S2Rs occurred. The latencies of these thousands of responses averaged 650–950 ms and were almost never shorter than 600. There is thus *a priori* reason to suspect that the 50 to 600 ms apparent S2R latencies in the above S1R-rich experiment (Fig. 7B) were not conventional responses to S2. A few flashes might have been spontaneous emissions that fell fortuitously within S1R or S2R latency ranges, but we believe most belonged to one or other of the following two effects, neither of which involved true S2Rs.

Because of the 400 ms minimal central nervous delay (Introduction) the short latency responses in Figure 7B could not have been evoked by S2s. Assuming that central delay was often longer than 400 ms, many could have been S1Rs. Thus (Fig. 8) any S1R excitation in which the centrally delayed fraction (CND) had been completed, and motor excitation (M) had started on its way to the lantern before the S2 signal arrived, would evoke a flash that could be misinterpreted as a short latency S2R.

The other phenomenon that almost certainly contributed to the spurious short-latency S2Rs of Figure 7B was the marked tendency for Female 62's flashes to occur in pairs about 1500 ms apart (Fig. 9). In about a third of the 90 such pairs recorded during more than two hours of unbroken driving, neither flash occurred less than 2 s after the preceding S2. By the criterion of Figure 7 such pairs were spontaneous. One such pair preceded each of phrases 4, 5, and 6 of Figure 6H and another is shown in Figure 9A. In another 30 instances either the first flash of the pair was spontaneous (*e.g.,* Fig. 6H, episodes 2, 6; 6I, episode 11; Figs. 9B–D) or the second was (Fig. 9I). In the remainder of the pairings, another 30 out of the 600 response total, it was not possible to distinguish between real S1Rs and flashes that happened to fall at a possible S1R latency (*e.g.,* 6A, episode 4; Figs. 9C–G). Similarly, S1RS2Rs (6C, episode 27; 6J, episode 17; 9E) were ambiguous in the sense that it is not excluded that the flash-pairing circuit might sometimes be activated by S1 rather than by the usual endogenous signal.

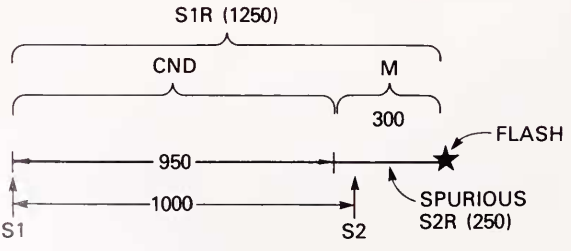

FIGURE 8. Proposed mechanism of misidentification of S1R as S2R. If excitation from S1 has completed its central nervous delay phase (CND) and triggered the motor outflow (M) to the lantern, a subsequent S2, before the flash, can be erroneously considered to be the effective stimulus.

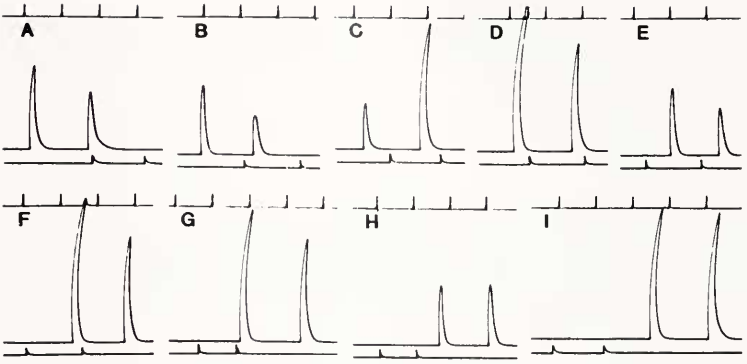

FIGURE 9. Flash pairs associated with paired signals of stimulus phrases. The types of association varied from one in which both firefly flashes were unequivocally spontaneous (SPSP; A), through instances in which the first flash was spontaneous (SPS1R; B, C, D) and the second not an S1R (B) or possibly so (C, D), to instances where both flashes might have been responsive (S1RS2R; E, F) or only the first (G, H), or probably neither (I).

Regardless of flashes of uncertain origin, the number of unequivocally spontaneous flash pairs, and the fact that they so often seemed independent of S1S2 scheduling and period, argue for flash pairing as a specific mode of female endogenous flash timing. Pair interflash durations for the entire record were heavily concentrated around a mean of about 1500 ms (22°) regardless of which of the 4 nominal classes of "response" was involved (Fig. 10). The validity of a spontaneous flash-pairing phenomenon was supported also by the flashing of one of the females in a slow driving experiment (Section 2, above). In this instance spontaneous flashing occurred throughout many of the 30 to 45 s rest periods between the isolated phrase presentations, much further removed from possible signal influence than with the 5 s and 10 s rhythms of the Figures 6–10 experiment, yet showed a pronounced 1500 ms (1.5 s) interflash peak (Fig. 11).

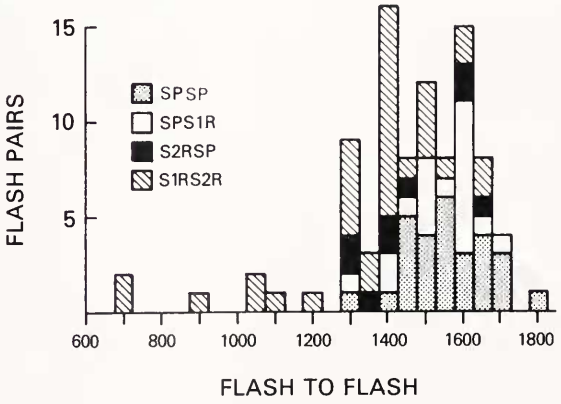

FIGURE 10. Frequencies of 90 interflash durations in four types of association of paired firefly flashes with paired signal phrases (*cf.* Fig. 9). The interflashes showed a strong independence from signal timing, suggesting spontaneity. Mean firefly interflashes: SPSP, 1530 ± 115 ms; SPS1R, 1543 ± 103; S2RSP, 1450 ± 135; S1RS2R, 1332 ± 243.

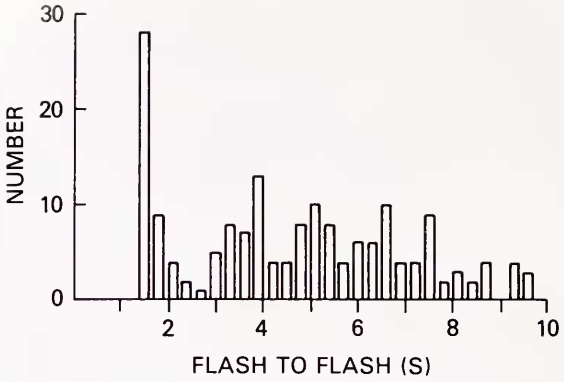

FIGURE 11. Frequencies of 124 spontaneous-flash-to-spontaneous-flash interval durations during about fifty 30 to 40 s intervals of non-stimulation, showing marked peak at about 1.5 s (1500 ms). Female 73.

In sum, though response overlap (Fig. 8) and flash pairing artifacts (Fig. 9) can account for the aberrantly short latencies of Figures 7A and 7B, and very probably some of the long latency tail of Figure 7B as well, photic stimulation of the female clearly can evoke a true response to the second stimulus of a phrase, after a delay of 700–950 ms at 22° (Fig. 7A) and sometimes also, or alternatively, a response to the first flash (or to a single stimulus flash) after a delay in the 800 to 1700 ms range, peaking at about 1250 ms (Fig. 7B). The S1R is thus not merely a premature S2R. How the two responses may be related mechanistically will be considered in the Discussion.

8. Further endogenous flashing types

In the field, neither unstimulated females nor those in dialog with either a male or with a flashlight were observed to flash spontaneously. Also, we recorded essentially no flashing during a 24-hour continuous box run with 4 unstimulated females. Enhanced spontaneity is probably a symptom of changing excitability induced by artificial driving, as is suggested also by the concurrently increased frequencies of flash skipping and of S1Rs. A major interest of spontaneity is that it supplements photic driving as an indicator of endogenous timing circuits. Flash pairing, for example, is clearly timed by an element with a relatively fixed duration averaging about 1500 ms at 21–22°. Evidence for other timed intervals or rhythms is more tenuous. The spontaneous flashes shown in Figure 6E suggest a rhythm period of about 5 s (mean of 6 interflashes = 4.8 ± 1 s), and the 4 spontaneous flashes interspersed between signal pairs 7 and 11 of Figure 6I divide that 40 s span into 8 intervals averaging 4.7 ± 0.4 s in duration.

Serially stimulated animals occasionally gave indication of endogenous luminescence at intervals in the 200–300 ms range. Usually this took the form of one full intensity flash preceded or followed by a much less intense shoulder, but occasionally the emissions were nearly equal in intensity (*e.g.*, Fig. 5H). In our opinion these emissions are not comparable to flash pairing. Rather, by reference to other fireflies, particularly during and after electrical driving (*e.g.*, Case and Buck, 1963; Buck *et al.* 1981A), they seem likely to be due to reverberation in a peripheral ganglion. Such short period emissions, and the fact that responses to rhythmic electrical driving may reach a frequency of several flashes per second (Case and Buck, in prep.), show that

whatever it is that sets the minimal 2.5 s S2 to S1 limit to re-excitation by rhythmic photic signals (Fig. 5D) it is not physiological inability to flash faster.

Discussion

Major flash timing patterns

Flash timing by *P. greeni* females results not only in the characteristic S2R response latency but in specific deviations related to photic history and stimulation regimen. When first exposed to paired flashes simulating the male's dialog code the female may not respond at all, then emit an irregular mixture of responses to the first signal of the pair (S1Rs), responses to the second (S2Rs) and flashes more than 2s later than the preceding stimulus (spontaneous flashes or SPs), then finally settle in to the repeated S2Rs of the natural dialog (Sect. 1; Fig. 2).

After warmup, many individuals continue 1:1 response for dozens or even hundreds of consecutive cycles if stimulus pairs recur at 15 s or longer intervals (Fig. 3). If pairs are presented more frequently, runs are typically shorter but still may include 1:1 rhythmic response (Fig. 5A, B). When the interval between successive pairs is 3.7 s or less, females no longer follow cycle by cycle, though they may respond at regular intervals (Fig. 5C, D). There may also be progressive falloff in flash intensity and increased frequency of response failures without change in stimulation format. Even during long stimulation series in which phrases are far enough apart to elicit 1:1 entrainment, response-skipping, S1Rs and SPs may develop, often alternating with runs of regular S1Rs or S2Rs (Fig. 6). A proper S1S2 interval is thus not enough in itself to assure indefinite rhythmic response.

S1R and S2R latencies differ in average duration (Fig. 7), and some apparent S1Rs and S2Rs are almost certainly spontaneous (Figs. 9, 10). There appear to be three specific categories of central nervous delay, associated with S1Rs (800+ ms), S2Rs (400+ ms) and endogenous flash pairing (*ca.* 1500 ms).

Types and causes of variability

There are several plausible reasons why identical stimuli did not always evoke the same response. Single cycle failures preceded and followed by dozens of consecutive responses (*e.g.,* Fig. 3, cycle 83, top trace) are presumably examples of the stochastic "noise" seen in most repetitive physiological events. In addition, latency, period and other response parameters of a given female of course exhibited statistical variation even during uniform rhythmic driving (Tables I, II).

Other types of response variation imply differences and changes in excitatory "state," a standard descriptive rubric for otherwise unexplained differences in responsivity to a given stimulation regimen. Thus the almost clocklike flashing of females stimulated in a slow rhythm (Fig. 3) illustrates a stable state of excitability lasting more than an hour. Alternating runs of S1Rs and S2Rs (*e.g.,* Fig. 6B–D) can be ascribed to an underlying cyclical variation in state. Females emitting sporadic spontaneous flashes may be presumed to be in a hyperexcited state. Fatigue is a likely cause of the progressive but reversible falloff in flash intensity during short-period rhythmic driving (Fig. 5D). The gradual decrease in response percentage that was common late in long rhythmic runs suggests a progressive falloff in general excitability, as in habituation. Functional recruitment during the warmup syndrome (Fig. 2) resembles arousal from the daylight torpor of *Photuris versicolor* (Case and Buck, 1963) which seemed to involve an actual physiological inability to flash and had to be overcome by me-

chanical agitation. It is presumably allied to the marked circadian rhythm of spontaneous flashing demonstrated in *Photinus pyralis* (Buck, 1937A).

One facet of excitation that was difficult to quantify was persistence of response for one or more cycles after stimulus parameters were changed, or delay in resuming response when it had been interrupted temporarily. In Figure 6E, for example, the female flashed at 5 s intervals for 6 or 7 cycles after the stimulation interval had been lengthened to 10 s. Here one could invoke "memory" of the prior driving rhythm, persisting for several cycles without reinforcement. A possibly analogous behavior has been reported by Carlson *et al.* (1977) for *P. macdermotti* in that "Females can be tricked into answering a signal pair, the signal interval of which [S1S2] is usually non-stimulating, by preceding the out-of-range pair with signal pairs within her acceptable range." Also if *P. macdermotti* females had responded to repeated 2-flash patterns and then were given only the first flash of the pair, they responded at the time corresponding to a normal S2R (Lloyd, 1984; see also below).

Finally, the records amply confirm the existence of much individual variation in type and in timing of both responsive and endogenous luminescences. Different females run simultaneously under essentially identical conditions sometimes differed in response latency by as much as 15% (Table I, II) and in response percentage by even more, confirming Carlson and Copeland's (1978) denigration of stereotyped uniformity in firefly behavior.

An uncontrolled factor in response variability was the presumably non-uniform population of subject fireflies, a difficulty we tried to minimize by making a large number of tests on many females. All animals used were responsive at the time of collection, but differences might still be expected in view of our indiscriminate sampling. Some females remained responsive for up to 10 days in the laboratory, and presumably in nature. Hence, even freshly gathered individuals may have spanned considerable ranges in age, health, and reproductive history.

In view of the sometime lability of light emission and the departures from dialog format, it is necessary to emphasize that the singly-flashed S2R is not only the nearly exclusive response under field courting conditions but also overwhelmingly the characteristic response during laboratory series. In many thousand box tests on hundreds of females, regular 1:1 S2Rs were the usual response to either isolated signal pairs or pairs repeated at a moderate rate. Anomalous flashing and breakdowns in entrainment were interesting for their possible physiological and behavioral implications (see below) but did not cast any doubt on the validity and generality of the standard courting code.

Flash control model

The responses evoked by photic stimulation raise the question of what sort of central nervous control system might be responsible. To explain the S2R the control must account for the following: (1) the flash-evoking S2 signal is preceded by an identical signal (S1) that has no visible effect prior to S2 but is nevertheless essential to the S2R; (2) the S2 must occur within a restricted range of intervals after the S1 (Fig. 4); (3) the female's response flash occurs only within certain time limits after S2 (Tables I, II); (4) after the S2R, the system cannot be re-excited for at least 2.5 s (Fig. 5D, S2s to next S1s). In addition, the control must accommodate the variations in behavior that have been ascribed to differing levels of neural excitatory state and to individuality. A system that meets most of these requirements is diagrammed in Figure 12A. For simplicity the model assumes that both the initial visual input link and the final motor outflow are all-or-none processes of fixed latencies that trigger downstream

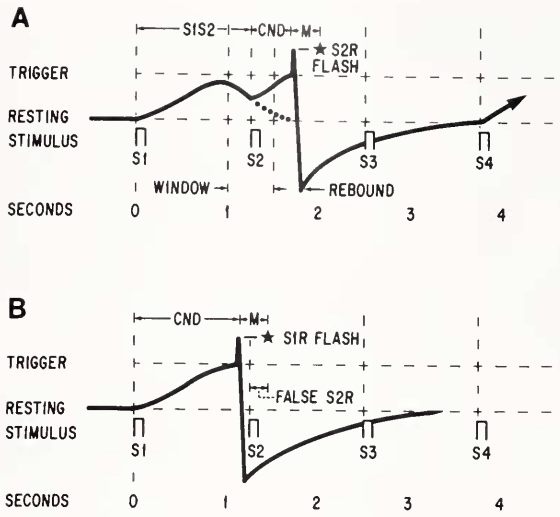

FIGURE 12. Model of female central nervous flash-control timer. A, S2R evocation. Excitation level rises after S1, then falls, enters the susceptibility window and rises again after S2, reaching the triggering level (at *ca.* 1750 ms) and exciting an S2R response. After the triggering spike, excitation level drops precipitously to a "hyperpolarized" level (REBOUND), from which it recovers so slowly that no excitation is elicited by S3. S4, arriving 2.5 s after S2, serves as a new primer for another response, after S5. S1S2 = 1250 ms; S2R = 800; CND = 500; M = 300; window = 500. B, S1R evocation. Excitation from S1 rises to triggering level at about 1100 ms, exciting a flash, then falls to a low level inexcitable by S2 or S3. The S1R flash at 1400 ms is also a spurious 150 ms S2R. S1S2 = 1250 ms; CND = 1100.

events but do not modulate the timing of light emission. The nominal 1250 ms S1S2 timing is a compromise between our mean preferred intersignal value at 23–25° (Fig. 4) and Buck and Buck's (1972) figures for 27°.

In the proposed model (Fig. 12A) some cellular excitation function (such as membrane potential or pulsatile activity within a group of central neurons) is represented as being at resting or basal level prior to S1. Upon S1 reception, excitation level begins to change but does not attain flash-triggering and falls back partway toward baseline (dotted line). After S1 the system is impervious to additional exogenous input for about 1s (Buck and Buck, 1972) then enters a time window, lasting about 500 ms, during which excitatory state is again susceptible to exogenous input. If S2 is presented during this window, excitation rises again, this time reaching the triggering level, thereby activating the motor signal to the lantern and evoking the S2R flash (star). Thus the S1 serves a trophic or priming function, raising excitation enough that an additional signal (S2) can achieve triggering. A female, after responding to S2, is refractory to photic input for at least 2.5 s (Fig. 5C, D). This could be accounted for if, after triggering, excitation falls below the resting level and occupies several seconds of central delay in recovering (Fig. 12A, "rebound").

Assuming that rate of increase in excitability can vary, the model provides plausible explanations for various departures from the standard S2R response. Flash skipping (*e.g.*, Fig. 2, episode 42; Fig. 3, episode 83; Fig. 6B, episodes 12, 22) would occur if excitation after the S2 did not rise to the triggering level. Similarly, if excitation after the S1 did not, as usual, peak and decline without reaching the triggering level (Fig. 12A) but continued to rise, an S1R could result (Fig. 12B). In that event, extra time would be occupied in the additional rise, and S1R latency would typically be longer

than S2R latency, which starts from a higher excitation level. In support, the average S1R incorporated about 400 ms more central nervous delay than did the average S2R (Fig. 7).

The model may also be compatible with certain non-dialog luminescences. A single spontaneous flash, for example, could be the analog of the S1R, with endogenous triggering in place of exogenous, or of the S2R, assuming that the endogenous timing control provides equivalents of both S1 and S2 signals. Spontaneous flash pairs, however, do not fit the model unless it is modified to provide (not unreasonably) that endogenous triggering does not induce the inhibitory undershoots proposed to follow response to external signals. Such a provision might also help explain the rarity of the two laboratory tandem events, S1RS2R and S2RS3R (Figs. 5G, 9, 10), already suspected of involving one or more spontaneous flashes.

The rate of rise of the transient should vary statistically and might also be subject to both endogenous and exogenous influences. Variations in response latency in the same female at different times and between different females run simultaneously (Tables I, II) should therefore be expected because the rates of charging and discharging of the timer would vary from cycle to cycle. The thresholds for basal and triggering excitation levels also would be expected to vary.

If the model is to accommodate the 300 ms latency to direct electrical eye stimulation (Introduction), it is necessary to envisage an immediate post-stimulus rise of excitation to the triggering level with consequent by-passing of central nervous delay.

In sum, an excitability transient operating between variable resting and flash-triggering limits (Fig. 12) provides a possible interpretation of the physiological control of the major female responses to photic input. Except as inherent in excitable membrane systems, the model does not supply specific physical bases for features such as the resting and triggering levels of excitability, the initiation and termination of excitation, or the post-S1 sensitivity window which is the key to the female's ability to count to two, *i.e.,* to recognize the male's characteristic flash pair code. In only one firefly has the flash-control area of the brain been even roughly localized, let alone analyzed (Bagnoli *et al.,* 1976, in *Luciola lusitanica*). Further elaboration of our model at this time would thus be gratuitous, particularly in view of the strong likelihood that flash control depends on a complex network of neurons rather than a single excitable cell. The model suggests an initial search for elements that are refractory for hundreds of milliseconds or more after excitation, and after flash triggering.

Behavioral comparisons, implications, and questions

Whatever the physiological basis of firefly laboratory flash timing phenomena, their behavioral significance in nature, and, *a fortiori,* their evolutionary implications, can only be established by exhaustive field observations. Such functional suggestions as we venture below are thus heuristic and tentative.

The sometimes marked individual differences in female flash-timing raises the question of how much variation is tolerated during the infrequently measurable natural dialog. Field data from several species show that the natural rhythm of the flying male's flashing varies in the same ranges observed in the present laboratory measurements on females (Buck, 1937B; Edmunds, 1963; Papi, 1969; Buck and Buck, 1972; etc.). It is also characteristic of firefly dialog that missed or mis-timed signals do not cause an immediate break in communication: males typically patrol the site of female answers for several cycles after response is interrupted, as it sometimes is by intervening foliage or by female failure to respond, and females often remain responsive indefinitely. It appears, therefore, that most timing variations measured in this study would not

prevent the *P. greeni* female response to the male's flash pair nor male recognition of her response latency. If for no other reason, a dialog pattern used in situations in which the body temperatures of the respondents might be as much as 2° different at their respective distances above the ground must allow considerable leeway in signal timing.

Consistent differences in response latency (Tables I, II) in different females conceivably could supply a basis for individual recognition (Lloyd, 1984)—if males are capable of the necessary discrimination.

In typical *Photinus* firefly courtships the male is the active advertiser and the female a responsive, not signal-initiating, partner. It is therefore not clear how spontaneous flashing by the female of *P. greeni,* if it occurs in the field, could function in communication. Speculation is unjustified until it is found whether the male ever responds to single flashes not timed to his S2, or to flashes paired at his own intra-phrase interval.

Lloyd (1969) and Carlson *et al.* (1976) described some *P. macdermotti* males as giving 2-2-2 second rhythmic single flashes when patrolling, and 2-4-2-4 second separated pairs when in dialog with a female or flashlight. This observation implies that the female normally responds both to unpaired flashes and to phrases and that the male is attracted to either answer. On the basis of still incomplete field observations and flight-cage experiments to be described elsewhere, the *P. greeni* male practically never emits unpaired flashes in flight but does sometimes approach S1Rs that follow several S2Rs from the same site. The S1R thus does not appear to play the systematic part in the *P. greeni* dialog that is reported for *P. macdermotti.* It might have utility in maintaining dialog in underbrush, where the male's S1 flash must sometimes be occluded (*cf.,* Carlson *et al.,* 1976; Lloyd 1984).

The fact that both males and females of *P. greeni* can emit pairs of spontaneous flashes deserves special comment. Usually, apparently, paired flashes are emitted only by the male. However, the female's response to the male's phrase is proof that her flash control system includes a timing element tuned to the S1S2. In view of the temperature coefficient of 2+ for flashing parameters (Materials and Methods), the female's mean 1500 ms intra-pair emission interval at 21–22° (Figs. 10, 11) and the male's field advertising rhythm at 27° (means for two individuals, 1260 and 1330 ms, Buck and Buck, 1972) should be close enough at a common temperature to suggest that they are generated by homologous timing circuits. Given that male and female are almost identical genetically it seems reasonable that in certain excitatory states the female's normally *sensory* timer could *initiate* flashes, as in the male. Somewhat analogous situations have been reported by Hoy *et al.* (1977) and by Doherty and Gerhard (1984) in the calling signals of crickets and frogs, respectively.

One additional gain from exploring the limits of flash-control is speculative insight into the evolution of firefly neural timing systems. Fascinating though such questions are, they are subject to the general caveat that any change in motor output-timing (male flashing period; female latency) would require concurrent change in the corresponding sensory input-timing of the other sex (Buck, 1978). Hence, simply being able to imagine a selective pressure for a code transition does not necessarily translate into a physiologically reasonable mechanism, or *vice versa.*

Lloyd (1984) reported that 13 late fall *P. macdermotti* females, if primed with signal pairs simulating the male's 2 s phrase, almost always responded to a single stimulus ("P1") after a delay of 3 s rather than at their normal 1 s latency to P2. From this evidence Lloyd proposed that the present courting code of *Photinus ignitus,* a close relative of *P. macdermotti* and *P. greeni* with a 3 s female response latency to the male's single signal, evolved by dropping out the second flash of the male's phrase

of a *macdermotti*-like ancestral species. The *P. ignitus* P1R would thus correspond not to the *greeni* S1R but to S1S2 + S2R. Assuming that *macdermotti* has the same flash-control system as *greeni,* Figure 12 suggests no obvious way to couple the female's S1S2 central delay to the equivalent of the S2R latency unless flashing at the end of the phrase-timing circuit can be triggered endogenously as well as by S2 (P2). In *P. greeni* we have not observed such a response, which would need to have a mean latency of at least 2 s, although we have not tested single signals as extensively as Lloyd.

Space does not permit discussing the three selection rationales proposed by Lloyd for the evolution of the *P. ignitus* code, but to us they seem no more persuasive than the view that evolution has proceeded in the opposite direction—that is, from codes in which the male emits a single signal to those in which he flashes in timed pairs. The selective advantage of a flash pair over a single flash would be the great increase in the female's ability to identify the male of the species. As shown by the ease of signal simulation with a wide variety of electric lights, neither flash intensity, flash duration, nor male advertising period offers a good basis for discriminating a conspecific single flash from singles of other species, or even other females. In that sense the *P. greeni* S1R might be the atavistic echo of a simpler past code, still latent in the nervous system.

In sum, exploration of *P. greeni* female responses under as natural conditions as compatible with controlled photic stimulation both confirmed the communicative pre-eminence of her response latency to the second flash of the male's courting phrase and yielded robust examples of systematic departure from standard dialog.

Regardless of whether our draft model proves to be correct in principle and whether particular timed intervals have a regular role in field behavior, the experiments revealed something of the range and potentialities of flash-control behavioral physiology. Spontaneous flashing, response skipping, S1Rs and other infrequent behaviors are *bonafide* capabilities of the intact nervous system. Some may serve only rarely in a communicative role, others may reflect response modalities used before the specialized dialog code had evolved to its present state, or indicate that similar underlying timing circuitry exists in the nervous systems of both male and female, as in the normal male and induced female emission at *ca.* 1500 ms intervals and possibly at the 5 s motor rhythm. Still others may be the physiological consequences of unusual input. Such responses, and the ability of the unrestrained, isolated female to produce spontaneous flashes, warn that not all nuances of light emission need be part of dialog. In fact there is good reason to expect that neural noise and fluctuations in excitability can and do normally provide a variety of occasional flashing behaviors that have no positive communicative significance.

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